Developmental Biology **209,** 298–307 (1999)

Ectopic *Msx2* Overexpression Inhibits and *Msx2* Antisense Stimulates Calvarial Osteoblast Differentiation

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***Msx2* is believed to play a role in regulating bone development, particularly in sutures of cranial bone. In this study we investigated the effects of retroviral-mediated overexpression of *Msx2* mRNA, in both sense and antisense orientations, on primary cultured chick calvarial osteoblasts. Unregulated overexpression of sense mRNA produced high levels of *Msx2* protein throughout the culture period, preventing the expected fall as the cells differentiate. The continued high expression of *Msx2* prevented osteoblastic differentiation and mineralization of the extracellular matrix. In contrast, expression of antisense *Msx2* RNA decreased proliferation and accelerated differentiation. In other studies, we showed that the *Msx2* promoter was widely expressed during the proliferative phase of mouse calvarial osteoblast cultures but was preferentially downregulated in osteoblastic nodules. These results support a model in which *Msx2* prevents differentiation and stimulates proliferation of cells at the extreme ends of the osteogenic fronts of the calvariae, facilitating expansion of the skull and closure of the suture.** © 1999 Academic Press

***Key Words: Msx2;* osteoblast; differentiation; homeodomain; bone; craniosynostosis.**

INTRODUCTION

Proper development of the skull requires precise control of proliferation and differentiation of osteoblast precursors to balance the need to allow growth to accommodate the expanding brain with the requirement for timely ossification. In these studies we have examined the role of the homeodomain-containing protein Msx2 in this process. *Msx2* is part of the *Drosophila msh* gene family, which includes vertebrate *Msx1, 2,* and *3.* While *Msx3* is primarily expressed in the central nervous system (Shimeld *et al.*, 1996; Wang *et al.*, 1996), *Msx1* and *2* are both expressed in numerous tissues at many stages of development (Davidson, 1995). Several studies suggest that *Msx2* plays a role in bone development. *MSX2* is expressed and regulated by vitamin D3 in human osteoblasts (Hodgkinson *et al.*, 1993) and inhibits the cotransfected type I collagen promoter in ROS 17/2.8 osteosarcoma cells (Dodig *et al.*, 1996; Towler *et al.*, 1994). Msx2 inhibits the osteocalcin promoter in transfected MC3T3E1 cells, but stimulates the same promoter in ROS 17/2.8 cells (Towler *et al.*, 1994). Msx2 and osteocalcin are expressed in a reciprocal pattern during tooth development (Bidder *et al.*, 1998). *Msx2* is strongly expressed in cells at the extreme ends of the osteogenic fronts of the calvarial sutures, and a gain-of-function mutation of *MSX2* causes Boston-type craniosynostosis (Jabs *et*

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0012-1606/99 $30.00

al., 1993; Ma *et al.*, 1996), a condition in which cranial sutures close prematurely. Overexpression of mutant or wild-type Msx2 driven either by the CMV promoter or by the mouse *Msx2* promoter also causes craniosynostosis (Liu *et al.*, 1995). In more recent studies, it was shown that overexpression of Msx2 driven by the mouse Msx2 promoter caused an increased number of proliferative osteogenic cells in the sutures of transgenic mice (Liu *et al.*, 1999). The studies described to date suggest an important role for *Msx2* in skull development; however, because Msx2 is expressed in both osteogenic cells and cells in the dura mater adjacent to the suture (Kim *et al.*, 1998), it is possible that Msx2 causes the release of factors from the dura mater which affect the osteoblastic cells of the suture. Thus we believe that it is important to determine the direct effect of Msx2 on osteoblastic cells.

To examine the effect of Msx2 on osteoblasts, we used the differentiating primary chicken calvarial osteoblast (cCOB) culture system, which provides a highly manipulable model of osteoblast development. Primary cCOB cultures show a pattern of bone marker expression and extracellular matrix maturation which is very similar to normal bone development (Gerstenfeld *et al.*, 1987). Exogenous genes can be stably integrated in essentially all the cells of a culture by infection with replication-competent retroviral vectors (Petropoulos and Hughes, 1991). In these studies, we showed that ectopic overexpression of Msx2 inhibited calvarial osteoblast differentiation *in vitro*. In contrast, antisense Msx2 RNA inhibited proliferation and causes premature differentiation of these cells. We also examined expression of a transgenic *Msx2* promoter-driven β-galactosidase construct in cultured calvarial osteoblasts, and we found that the transgene was specifically downregulated in differentiating osteoblasts. These results suggest that one of the normal biological roles of *Msx2* is to inhibit differentiation and stimulate proliferation in preosteoblastic cells in the osteogenic front of the sutures, thereby facilitating expansion of the skull bones and closure of the sutures.

MATERIALS AND METHODS

Primary Chick Calvarial Osteoblast Culture

Chick calvarial osteoblasts were isolated from 15-day-old chick embryonal calvariae as described in Gerstenfeld *et al.* (1987) by four sequential 15-min digestions in 0.05% trypsin (GIBCO, Grand Island, NY) and 0.1% collagenase P (Boehringer Mannheim) at 37°C on a rocking platform. Fractions 2–4 were collected and plated at 5000 cells/cm^2 in six-well culture plates in DMEM containing 20% FCS. The medium was changed to DMEM containing 10% FCS 24 h later. After the cells became confluent, differentiation medium (BGJb, Fitton–Jackson modification; GIBCO; containing 10% FCS, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate) was used to maintain the cells for the duration of the experiment. Cells were harvested for analysis at different stages of differentiation.

Primary Mouse Calvarial Osteoblast Culture and β-Galactosidase Staining

Primary mouse calvarial cells were isolated as described in Dodig *et al.* (1996), using a modification of the method of Wong and Cohn (1974). Briefly, calvariae from 6- to 8-day-old transgenic mice containing the Msx2 promoter driving the *Escherichia coli LacZ* gene (Liu *et al.*, 1994) were dissected free of sutures and adherent tissue and then digested with trypsin–collagenase as described for chick calvaria. Five fractions were collected; the first two were discarded and fractions 3–5 were pooled, plated at 10^4 cells/cm^2, and cultured in DMEM with 10% fetal bovine serum. After the cells reached confluence, the medium was changed to αMEM with 25 μg/ml ascorbate and 5 mM β-glycerophosphate to allow differentiation. β-Galactosidase staining was carried out using a protocol given to us by Dr. Alexandra Joyner. This protocol is essentially as described in Logan *et al.* (1993), with the exception that 0.1 M $NaPO_4$, pH 7.3, was used instead of phosphate-buffered saline, and 0.1% Na deoxycholate was used in the stain and wash buffers.

Retroviral Vectors and Infection of Chick Primary Osteoblast Cultures

Full-length chicken *Msx2* cDNA was cloned into the *Cla*I site of the RCASBP(A) helper-independent retroviral vector (Petropoulos and Hughes, 1991) in the sense and antisense orientation. As a control virus we used an RCASBP(A) vector without *Msx2* cDNA. Vectors were transfected into producer cells (chicken embryonal fibroblasts (CEF)) using the calcium phosphate method. CEF conditioned medium with high reverse transcriptase activity, measured by the procedure described in Petropoulos and Hughes (1991), and a titer estimated at $1–3 \times 10^8$ infectious particles per milliliter (Ferrari *et al.*, 1998) was collected and stored at −70°C until use. cCOBs were infected with 0.5 ml of conditioned medium containing RCASBP(A)-Msx2, RCAS-Msx2 As, RCAS, or no virus (mock) on 3 successive days beginning with the first day after the cells were plated.

Assessment of Mineralization

Mineralization of cultures was determined by von Kossa staining (Page, 1982).

Immunostaining for Msx2

Cells were fixed in ice-cold 95% ethanol for 1 h, followed by 1 h in ice-cold 100% methanol and overnight postfixation in 95% ethanol. Cells were then treated with 100% ethanol:glacial acetic acid (95:5) for 5 min and then dehydrated with four changes of ice-cold 100% ethanol. Cells were washed with phosphate-buffered saline three times for 5 min each and incubated with a polyclonal rabbit antibody against chicken Msx2 produced and characterized as previously described (Ferrari *et al.*, 1998). The cells were then incubated with cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescent black and white 40× images were photographed using Kodak Tekpan ASA 100 film and a Nikon Optiphot microscope with a rhodamine filter and digitized using a slide scanner. Color 4× images were photographed using a Diagnostic Instruments Spot cooled CCD digital camera. For both types of image, all cell types were photographed using the same exposure conditions. Both types

of image were arranged and labeled and intensity levels adjusted using Adobe PhotoShop. All adjustments were made on all images of a group simultaneously, so that no changes in relative intensities of different images were introduced. Images were printed using a Kodak dye sublimation printer.

Isolation and Analysis of RNA

RNA was isolated using TRI reagent (Molecular Research Center, Inc.) (Chomczinski, 1993). Ten micrograms of total RNA was separated on a 1% agarose 1.1 M formaldehyde gel and transferred to nylon membrane (Extra Strength Nytran; Schleicher & Schuell). Probes for chick *Msx2* (Coelho *et al.*, 1991), osteocalcin (Neugebauer *et al.*, 1995), osteopontin (Rafidi *et al.*, 1994), bone sialoprotein (BSP) (Yang *et al.*, 1995), and alkaline phosphatase (Crawford *et al.*, 1995) were full-length cDNAs cloned into the *Eco*RI site of pBS (Stratagene, La Jolla, CA). The probe for chick α1(I) collagen was isolated from the clone pcg54 (Finer *et al.*, 1987). The cDNA fragments were gel purified using SpinBind DNA purification columns (FMC BioProducts, Rockland, ME) and random primer labeled (Feinberg and Vogelstein, 1983). Hybridization was performed in 50% formamide and 6× SSPE at 42°C (Sambrook *et al.*, 1989). Hybridization to 18S RNA probe or inspection of ethidium bromide-stained filters was used to evaluate loading of different lanes.

Assessment of Cell Proliferation

Cell number of 5-day-old cultures was determined by trypsinizing the cells from the plate and counting in a hemacytometer using three independent samplings per plate.

For quantitation of dividing cells, 4-day-old cultures were incubated in 20 μM BrdU (Sigma) overnight at 37°C. BrdU-positive cells were visualized using a protocol derived from published methods (Gray, 1985). Cells were fixed with 10% formaldehyde, permeabilized and denatured with 2 M HCl in 0.1% Triton X-100 for 30 min at 37°C, and washed with 0.1 M sodium borate, pH 8.5. Cells were incubated with monoclonal anti-BrdU antibody (Sigma) diluted 1:1000 in PBA for 90 min, followed by incubation with TRITC-conjugated anti-mouse IgG (Sigma). After excitation at 546 nm cells with incorporated BrdU were visualized, photographed, and counted in 10 random fields using a fluorescence microscope (Zeiss Axiovert 135) at 10× magnification.

DNA content of 5-day-old cultures was measured by extracting DNA using the TRI reagent (Molecular Research Center, Inc.) (Chomczinski, 1993) and measuring the absorbance at 260 nm.

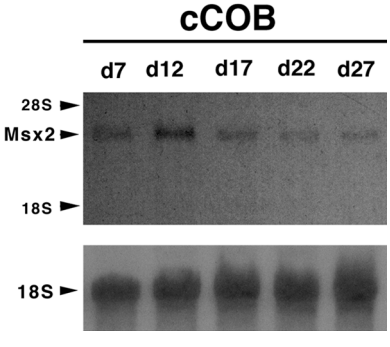

FIG. 1. *Msx2* mRNA expression during chick calvaria osteoblast differentiation. Numbers indicate days after plating. Cultures begin to differentiate after day 7. Hybridization to 18S RNA is shown as a loading control.

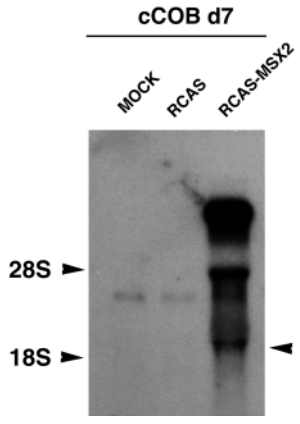

FIG. 2. RCASBP(A)-Msx2-mediated expression of *Msx2* in chick calvarial osteoblasts. Northern blot analysis of osteoblast cultures treated with chick embryo fibroblast conditioned medium (mock), control RCASBP(A) virus (RCAS), and RCASBP(A)-Msx2 (RCAS-Msx2). The band in lanes labeled MOCK and RCAS is the endogenous *Msx2* transcript. The arrowhead indicates the only retrovirus transcript which can be translated to produce *Msx2* protein.

RESULTS

To study the role of *Msx2* in osteoblast development, we used the chick calvarial osteoblast culture system. Northern blot analysis reveals that *Msx2* expression in differentiating cCOB is highest in early cultures (days 7 and 12), while in the more differentiated cultures (days 17–27) *Msx2* mRNA levels are significantly reduced (Fig. 1). The increase seen between day 7 and day 12 was not always seen; however, the decrease seen in later cultures was very reproducible. This pattern corresponds to that seen in differentiating murine calvarial osteoblast cultures.

To test whether constitutive overexpression of *Msx2* would have an effect on calvarial osteoblast differentiation, we infected chicken primary osteoblast cultures with a replication-competent retrovirus expressing Msx2 (RCASBP(A)-Msx2). Osteoblastic differentiation was determined by Northern blot analysis using probes for mRNAs which are characteristically present at high levels in differentiated osteoblasts. Alkaline phosphatase is the earliest marker to be induced during osteoblastic differentiation; osteopontin is generally induced somewhat later, and osteocalcin induction is the latest marker to be induced (Aubin and Turksen, 1996). We also probed for type I collagen, which, although it

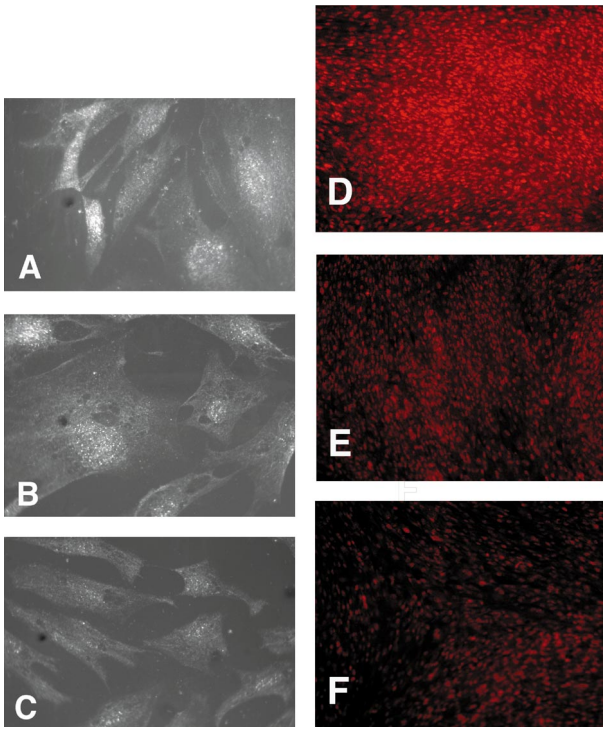

FIG. 3. Immunohistochemistry detecting Msx2 protein in RCASBP(A)-Msx2-infected (A, D), control (B, E), and RCASBP(A)-Msx2 antisense-infected (C, F) chick calvarial osteoblasts. A–C are images taken with a 40× objective of cells replated at low density to allow visualization of individual cells. D–E are images taken with a 4× objective of primary cells at high density to allow visualization of overall intensity of staining. Phase-contrast visualization of these cultures indicated that the three types of cells were at similar densities.

is expressed in osteoblast precursors, is induced to high levels during differentiation. Although some reports indicate that type I collagen expression is highest in the early proliferative phase of osteoblast culture (Owen *et al.*, 1990), our studies have found that this marker is highest in post-proliferative differentiating cultures (Dodig *et al.*, 1996 and unpublished results). Control experiments demonstrated that exposure of cCOB cultures to chick embryo fibroblast conditioned medium (mock infection) or to RCASBP(A) virus had no effect on differentiation (data not shown). *Msx2* overexpression in infected cCOB cultures was examined by Northern blot analysis of *Msx2* mRNA (Fig. 2). The arrowhead indicates the spliced retroviral RNA which is the only RNA species which can be translated to produce *Msx2* protein. The larger RNAs have upstream reading frames which encode the viral gag, pol, and env proteins and thus cannot translate the Msx2 cistron (Petropoulos and Hughes, 1991). In addition, immunostaining using polyclonal *Msx2* antibodies indicated that most if not all of the cells in the RCASBP(A)-*Msx2* infected cultures (Figs. 3A and 3D) had strongly staining nuclei, in contrast to the control cultures (Figs. 3B and 3E), which were more lightly stained, reflecting the moderate expression of endogenous *Msx2*. The proliferation and appearance of undifferentiated 5-day-old cultures was not affected by *Msx2* (Table 1 and data not shown). To determine the effect of *Msx2* on differentiation into mature osteoblasts, we examined the expression of osteoblastic markers in the cultures by Northern blot analysis. Expression of type I collagen and osteopontin was decreased (Fig. 4A), and alkaline phosphatase and osteocalcin in the 28-day-old cultures were barely detectable (Fig. 4B), compared with control cultures infected with RCASBP(A) or mock-infected controls. The most dramatic morphological effect of RCASBP(A)-Msx2 infection was the absence of mineralization of the extracellular matrix, while mineralization was demonstrated by both mock and RCASBP(A) control cultures (Fig. 4C). These experiments were repeated three times with very reproducible results in each case.

In a second series of experiments we engineered a retrovirus expressing *Msx2* RNA in the antisense orientation (RCASBP(A)-Msx2As). Immunostaining with Msx2 antibodies demonstrated decreased levels of Msx2 protein in antisense-treated cultures compared to controls (compare Figs. 3C and 3F to Figs. 3B and 3E) Differentiation of antisense-infected cultures was accelerated as shown by earlier expression of bone markers. Type I collagen mRNA in *Msx2* antisense-infected cultures was increased compared to RCASBP(A)-infected cultures on day 7, indicating that the Msx2 antisense has accelerated the normal differentiation of these cultures. By day 12 collagen mRNA levels were similar in controls and antisense-treated cultures (Fig. 5A). This is consistent with our observation that endogenous levels of Msx2 are decreasing during this time period,

TABLE 1

Effect of Msx2 Sense and Antisense on Cell Proliferation

	RCAS	Msx2As	Msx2
Cell number			
Expt 1	38.7 ± 0.7 (3)	29.3 ± 1.4 (3)*	38.0 ± 0.6 (3)
Expt 2	19.3 ± 2.0 (3)	15.3 ± 1.2 (3)*	20.3 ± 0.3 (3)
Expt 3	35.0 ± 1.7 (3)	26.3 ± 0.3 (3)*	35.0 ± 1.5 (3)
DNA content			
Expt 1	11.8 μg	8.5 μg	11.5 μg
Expt 2	16.3 μg	9.5 μg	13.8 μg
BrdU incorporation	94.1 ± 7.4 (10)	72.0 ± 2.7 (10)*	94.1 ± 7.4 (10)

Note. Cell number and BrdU incorporation are shown as the mean ± standard error of the mean, with the number of replicates in parentheses. DNA content was determined by quantitation of DNA in 5 or 6 35-mm dishes, then calculation of the amount of DNA per dish. All assays were done on 5-day-old cultures.

* Values significantly different from RCAS values at $P < 0.05$.

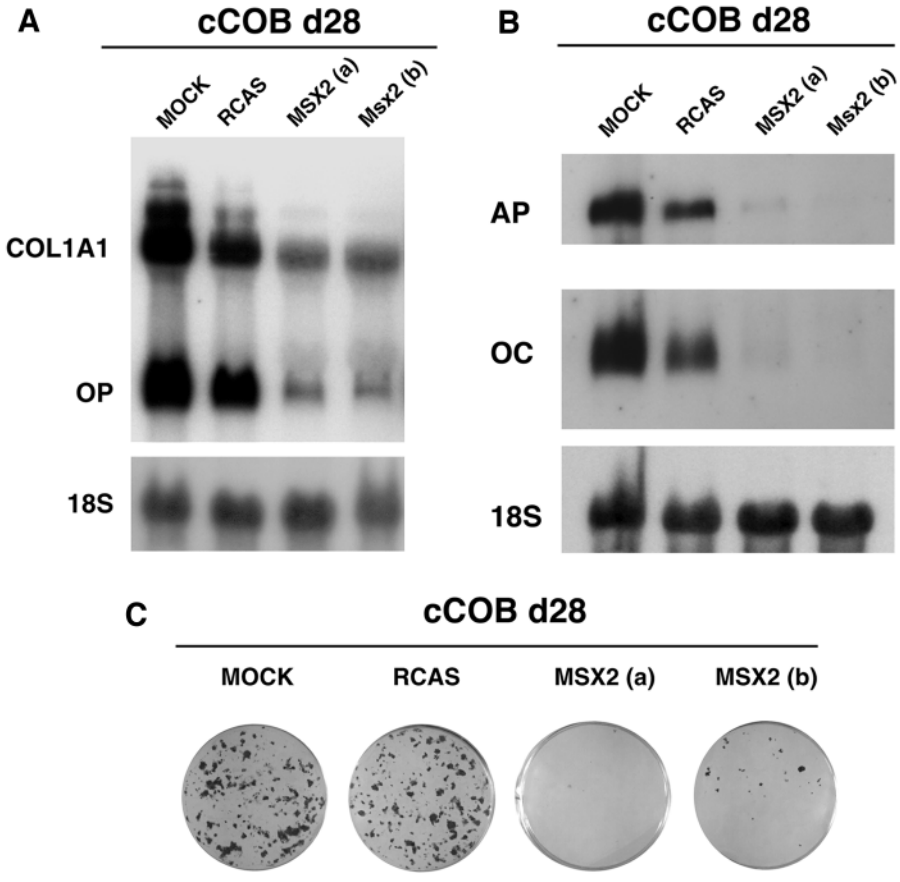

FIG. 4. Effect of *Msx2* on osteoblast markers in 28-day osteoblast cultures. (A) Effect of *Msx2* on Col1a1 and osteopontin (OP) mRNA. (B) Effect of *Msx2* on alkaline phosphatase (AP) and osteocalcin (OC). (C) Effect of *Msx2* on mineralization as revealed by von Kossa staining. Black spots are areas of mineralization. Labels are as in Fig. 3 caption. *Msx2* a and b are duplicate experiments.

allowing increased expression of type I collagen, which is a relatively early marker of differentiation. Osteopontin mRNA was slightly induced on day 7 and was strongly induced on day 12, when very little endogenous mRNA was present in the control cultures. Alkaline phosphatase was induced relative to control on days 7 and 12. Osteocalcin was strongly induced on day 12 of culture, when the control cultures showed very minor induction of this mRNA. Mineralization of the extracellular matrix was also strongly induced compared to control in day 12 cultures (Fig. 5C). These observations, which were consistently seen in three separate experiments, support the conclusion of the *Msx2* overexpression studies, suggesting that endogenous *Msx2* inhibits osteoblastic differentiation during the early proliferative phase of *in vitro* osteoblast differentiation.

Msx2 antisense-infected cultures appeared to be less proliferative than control cultures. Since proliferation is often inversely correlated with differentiation, we examined three indices of proliferation in three separate experiments comparing *Msx2* antisense and control cultures. Cultures infected with RCASBP(A)-Msx2As demonstrated 25–30% fewer cells, less DNA per plate, and a reduced number of BrdU-positive cells compared with cultures infected with control virus (Table 1), indicating that endogenous *Msx2* stimulates proliferation in calvarial osteoblastic cells.

Although previous studies showed that *Msx2* mRNA expression decreases during mouse calvarial osteoblast differentiation (Dodig *et al.*, 1996), *Msx2* mRNA levels were still substantial at a time when the cultures were undergoing differentiation. It could not be determined using these Northern blot studies whether *Msx2* decreased in all cells in the culture at the same rate or if *Msx2* decreases more rapidly in osteoblastic nodules than in surrounding nonosteoblastic cells. To address this question, we used transgenic mice containing 5.3 kb of the *Msx2* gene 5′ flanking

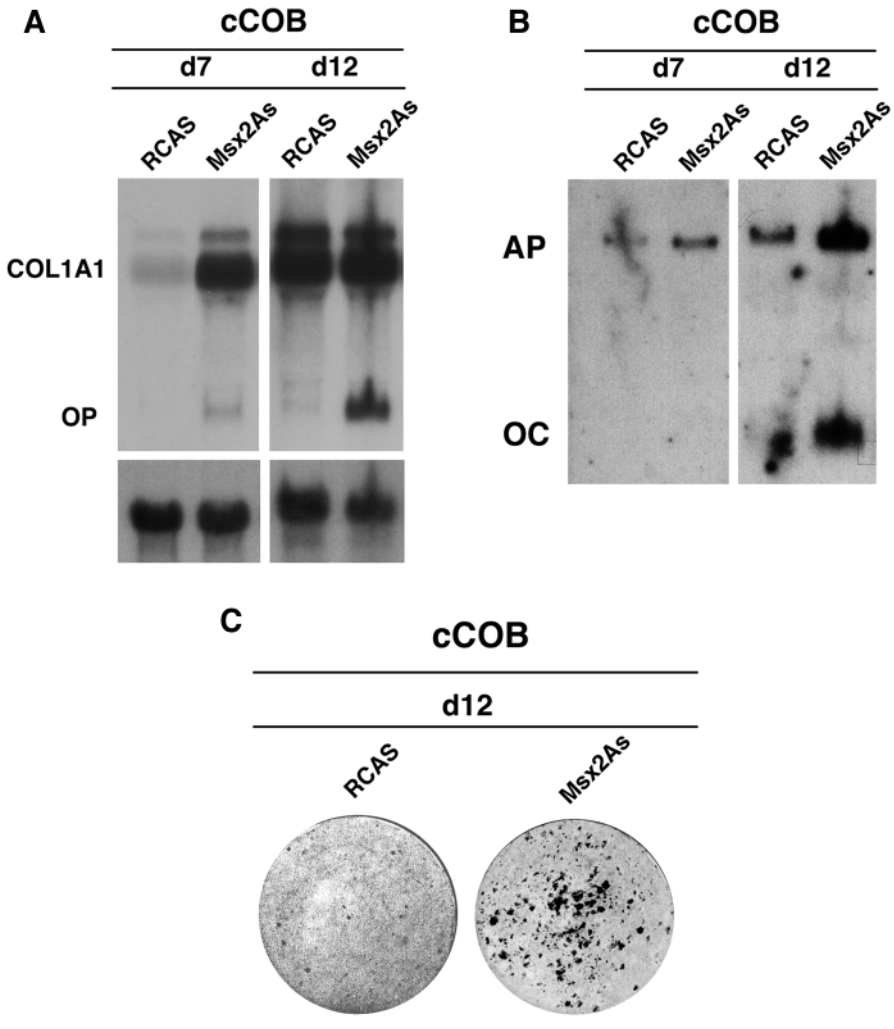

FIG. 5. Effect of *Msx2* antisense (Msx2As) on osteoblast differentiation after 7 (d7) or 12 (d12) days in culture. (A) Effect of Msx2As on Col1a1 and osteopontin (OP) mRNA levels. (B) Effect of *Msx2* on alkaline phosphatase (AP) and osteocalcin (OC). B is a rehybridization of the blot shown in A, so the 18S normalization applies here as well. (C) Effect of *Msx2* on mineralization as revealed by von Kossa staining. Black spots are areas of mineralization.

sequence driving β-galactosidase. Previous studies (Liu *et al.*, 1994; 1999; and our unpublished observations) have shown that this transgene is expressed in the calvarial sutures in a pattern similar to that of the endogenous gene. Calvarial osteoblasts from these mice were cultured under conditions which allow *in vitro* differentiation and stained for β-galactosidase at various stages. Although sutures were removed from the calvariae, so that most of the plated cells would not have expressed *Msx2 in vivo*, by day 1 after plating most of the cells were beginning to express the transgene (Fig. 6A), and by day 4 almost all cells expressed β-galactosidase (Fig. 6B). By day 11, when osteoblastic nodules had begun to form, β-galactosidase was restricted to cells between the nodules (Fig. 6C). By day 16 β-galactosidase was restricted to the cells immediately surrounding the nodules (Fig. 6D). This pattern of expression is consistent with the hypothesis that *Msx2* inhibits osteoblastic differentiation.

DISCUSSION

In these studies we examined the role of *Msx2* in skull bone development. In order to directly assess the function

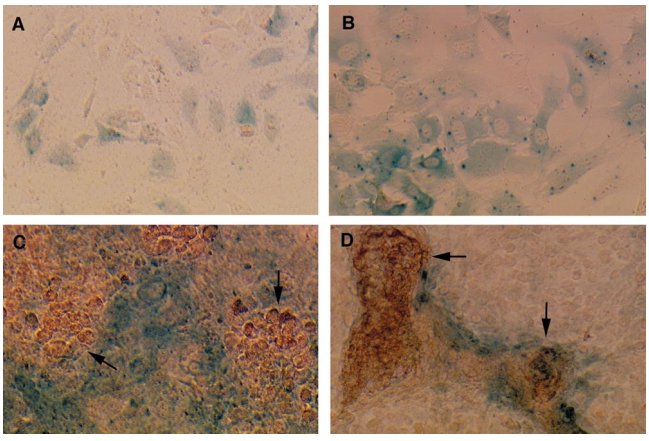

FIG. 6. *Msx2* promoter-driven β-galactosidase transgene expression during calvarial osteoblast development. Calvarial osteoblasts from 6- to 8-day-old transgenic mice containing −5.2/lacZ were cultured for 1, 4, 11, and 16 days (A, B, C, and D, respectively) and then stained for β-galactosidase activity. Arrows in C and D point to osteoblastic nodules.

of *Msx2* in this process, we forced continued expression of *Msx2* in calvarial osteoblasts beyond the stage at which the endogenous gene is normally downregulated. We found that osteoblastic differentiation was strongly inhibited. Antisense inhibition of *Msx2* expression stimulated differentiation and decreased proliferation, further supporting the role of endogenous *Msx2* in inhibiting differentiation and suggesting that *Msx2* also stimulates proliferation. Overexpression of Msx2 did not appear to stimulate proliferation. This may be because the endogenous levels of Msx2 present in the cultures coupled with serum growth factors may have stimulated maximal proliferation, such that higher levels of Msx2 were not able to stimulate increased proliferation.

Previous studies had indicated that *Msx2* expression decreases as calvarial osteoblast cultures differentiate. To investigate this observation in greater detail, we used transgenic mice containing an *Msx2* promoter-driven β-galactosidase transgene. We found that this transgene is expressed in calvarial cells cultured *in vitro,* despite the fact that the sutures and outer cells of the periosteum, which contain the vast majority of *Msx2*-expressing cells in the calvaria (Jabs *et al.*, 1993), were removed before isolation of the cells which were plated for the experiments. This observation is consistent with previous studies showing that endogenous *Msx2* mRNA is expressed in cultured mouse calvarial cells but not in calvariae with the sutures and part of the periosteum removed (Dodig *et al.*, 1996). The induction of *Msx2* in cells which did not express *Msx2 in vivo* suggests the possibility that all calvarial osteoblasts may be capable of expressing *Msx2 in vivo* and that a specific signal induces *Msx2* in the cells in the suture area. Alternatively *Msx2* expression may be constitutive in calvarial osteoblast precursors, but may be suppressed in cells outside the suture. The second possibility is consistent with the fact that *Msx2* is expressed in migrating cranial neural crest cells, which include the precursors of calvarial osteoblasts. It appears that the conditions allowing expression of *Msx2* are mimicked by *in vitro* culture. Analysis of β-galactosidase expression during *in vitro* differentiation of these cultures indicated that the *Msx2* transgene was downregulated first in the osteoblastic nodules and only later in the internodular cells. This observation further supports the hypothesis that *Msx2* inhibits osteoblastic differentiation.

Our results, combined with previous studies on *Msx2*, can be used to construct a model for the role of *Msx2* in calvarial bone development and to explain why increased *Msx2* function causes craniosynostosis (Fig. 7). *Msx2* is expressed primarily in cells at the extreme end of the osteogenic front of the suture and in cells in between the sutures. There is decreased or absent expression in the cells flanking the front. We believe that *Msx2* expression constrains the committed preosteoblastic cells in the osteogenic front in an undifferentiated stage and probably stimulates proliferation to some degree, maintaining a zone of rapid lateral bone growth at the edge of the calvariae (Fig. 7A). Downregulation of *Msx2* expression is necessary for the progression of these cells further into the osteoblastic lineage. The *Msx2* gain-of-function mutation found in Boston-type craniosynostosis (Jabs *et al.*, 1993), or increased expression of normal *Msx2* in transgenic mice (Liu *et al.*, 1995), may lead to an expansion of the zone of undifferentiated, proliferating cells which make up part of the osteogenic front (Fig.7B). The increase in the size and growth rate of this group of cells disrupts the synchronization between calvarial bone growth and expansion of the brain, which is needed to maintain the proper distance between the skull bones, therefore the osteogenic ridges from neighboring calvarial bones meet prematurely (Fig. 7C). In studies on the calvariae of Msx2-overexpressing transgenic mice, it was found that the osteogenic fronts of mice expressing Msx2 from the Msx2 promoter show enhanced growth and increased BrdU labeling compared to those of control mice, consistent with our hypothesis (Liu *et al.*, 1999). We hypothesize that in the human the juncture of two osteogenic fronts triggers downregulation of *Msx2,* allowing osteoblastic differentiation and final closure of the suture. In the mouse, fusion of the sutures does not occur in 4-month-old adult mice (Zimmerman *et al.*, 1998), therefore Msx2 may not be downregulated. Mice expressing *Msx2* driven by the CMV promoter had normal ossified bone (Liu *et al.*, 1995). Based on the results of the current studies, we would predict that this would not

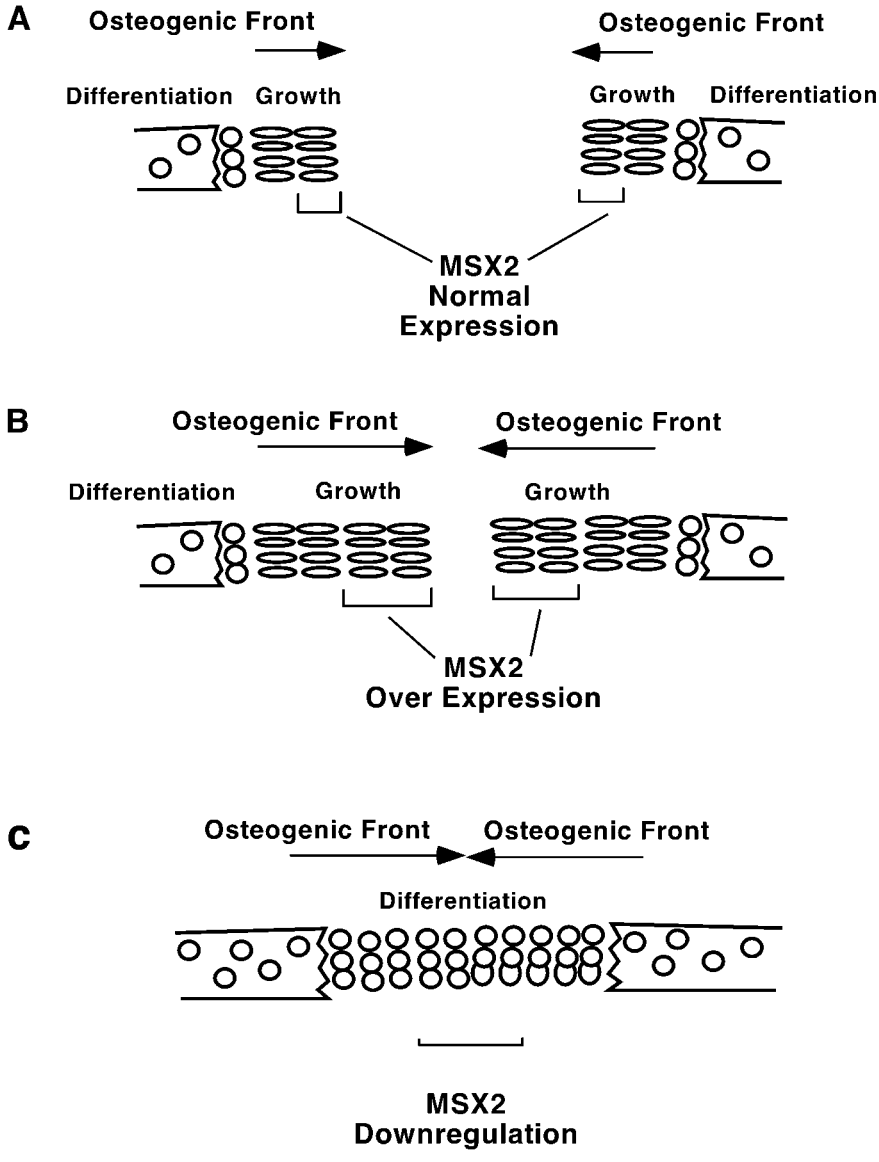

FIG. 7. Model of the role of *Msx2* in normal skull development and the role of its overexpression in craniosynostosis.

occur if the *Msx2* gene was expressed in mature osteoblasts.

The role that we have proposed for *Msx2* in modulating cell division and differentiation of specific preosteoblastic cells is consistent with the effects of the "selector" homeoproteins on *Drosophila* development, which include modulation of rates of cell division and differentiation into specific cell types (Biggin and McGinnis, 1997).

Winograd *et al.* (1997) have analyzed transgenic mice containing the human *MSX2* gene and have found an extremely severe phenotype characterized by perinatal lethality and multiple craniofacial malformations, often characterized by loss of skeletal structures. This apparent discrepancy with the results of Liu *et al.* (1995) may be because multiple integrated copies of the entire human *MSX2* gene locus produced much higher levels of MSX2 protein than the transgene used in the studies of Liu *et al.*, or *MSX2* may have been expressed ectopically. The pattern and levels of expression of the MSX2 protein were not assessed by Winograd *et al.*, 1997.

Our model predicts that inactivation of the *Msx2* gene would cause delayed closure of the cranial sutures. Although an *Msx2* gene knockout mouse has been described (Maas and Bei, 1997), the time of closure of the cranial

sutures was not reported. It will be of interest to examine suture closure in further studies of this mouse.

ACKNOWLEDGMENTS

We thank Dr. R. Kosher, C. Dealy, and D. Ferrari for providing us with the *Msx2* antibody and for the immunohistochemistry protocol. We also thank Dr. Stephen Hughes for giving us the RCASBP(A) vector and for many helpful suggestions concerning its use. This work was supported by the following grants from the National Institutes of Health: AR29983 (A.C.L.), HD22610 (R. Rosher and A.C.L.), and AR38933 (A.C.L. and D.W.R.).

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Received for publication November 25, 1998
Revised February 26, 1999
Accepted February 26, 1999